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Effect of Thermal Stress across Development in Drosophila melanogaster

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Abstract:

Animal development is a complex process that requires successful completion of multiple steps at different developmental stages to produce adult organs and systems. Environmental stress experienced during crucial developmental stages could therefore disrupt the proper functioning and survival of individuals as adults long after the stressor has passed. Early embryonic stages may be particularly susceptible to long-lasting effects because cellular mechanisms of stress resistance are relatively underdeveloped. In chronically cold or hot environments, such stress may impose significant natural selection on early embryonic developmental systems to improve developmental resilience in the face of temperature extremes.

In this study, I tested the impact of thermal stress on development in the fruit fly *Drosophila melanogaster,* for which the sequence of development has been well-described and is known to experience significant heat stress during embryogenesis in the field. I asked two main questions: 1) Are early embryonic stages of development more sensitive to thermal stress than later developmental stages, and 2) have hotter climates resulted in adaptive resilience to thermal stress during early embryonic development? To test whether early embryos are more sensitive to thermal stress, I compared survival, morphological and performance metrics of flies of the Canton-S strain exposed to cold or heat stress at 1, 24, or 60 hours in development. To test whether high temperatures result in adaptive resilience, two tropical and two temperate populations of *D. melanogaster* from around the globe were tested. The tropical populations originated from Chiapas, Mexico and Guam, and the temperate populations both originated from northern Vermont. The eggs of these populations were reared at 25°C for 1 hour before being transferred to 18°C, 25°C, 30°C, and 32°C incubators and tested to see if they also showed defects seen in Canton-S flies under thermal stress. I found that later developmental stages acclimated better to moderate thermal stress and incurred fewer lasting phenotypic consequences because of that thermal stress compared to early developmental stages. Early embryos experienced a high proportion of deformed wings and many of the pupae failed to eclose into adults. Twenty four-hour flies were found to have a greater proportion of properly developed wings, eclosed from pupae into adults at a higher proportion, and displayed superior upper and lower thermal limits than 1-hour flies.

When testing for thermal adaptation during early development between tropical and temperate populations, I found substantial variation between the tropical populations, with only the Chiapas population displaying evidence for thermal adaptation. Chiapas routinely performed better in eclosion success, climbing success, and CTmax. The Guam population, however, frequently performed equally or worse than the temperate populations. Thus, thermal adaptation during development may not have acted equally or even similarly on populations from the same climate.

Both parts of this research have important implications for the future of *D. melanogaster* populations as climate change will continue to affect daily and seasonal temperatures for many *D. melanogaster* populations. Because flies in early embryonic development are highly sensitive to moderate thermal stress, *D. melanogaster* populations need to have sufficient adaptive potential to adapt to changing climates during early development. Results from the Chiapas population illustrate how thermal adaptation during early development can buffer populations against moderate thermal stress, possibly allowing populations of *D. melanogaster* around the globe to adapt to hotter temperatures that arise from climate change.

Introduction:

The world is full of stressors. Species may have to fend off predators, go long periods of time without food, contract diseases, or live-in unideal climates. The landscape of thermal stress, in particular, will continue to change as climate change progresses. Global average temperatures due to global warming are expected to increase by 7°C by the year 2100, increasing the chance populations experience temperatures close to their upper thermal limit, making it important to understand the impact of thermal stress on survival and performance of natural populations (Sherwood and Huber, 2010).

Most species on Earth share evolutionarily conserved biological systems that allow them to identify stress on the cellular level and respond accordingly (Huang et al. 2021). The most common and conserved mechanism for how species responds to thermal stress is mobilization of heat-shock proteins (HSPs) (Stephanou et al. 1982). HSPs are a family of chaperone proteins that are expressed in the face of heat or other stresses and work by minimizing aggregation of stress-damaged proteins and targeting them for repair or excision (Roberts and Feder, 1999). There are several different HSPs involved in the heat shock response, with Hsp70 being known to be involved in mitigating the effect of hyperthermia during development (Roberts et al. 2003).

One of the most well-studied organisms regarding mechanisms of thermal stress and heat shock is the fruit fly *Drosophila melanogaster*. *Drosophila melanogaster* is a model organism for such studies because they have a small genome that is well understood, short generation time, and produce large numbers of offspring. *Drosophila melanogaster* typically lays its eggs in necrotic fruit, and simply being exposed in unshaded fruit to the sun can cause flies to experience hyperthermia (Roberts and Feder, 1999). They also have a global distribution, so natural populations are expected to vary in the extent and duration of thermal stress events.

Organisms born into adverse conditions may experience a variety of stressors early in life that could affect their development and fitness. During *D. melanogaster*'s development, many structures and biological systems have important developmental stages when crucial steps are undertaken to ensure proper growth and functioning as adults (Tyler, 2000). These stages can be disrupted in response to thermal stress. Early development is particularly vulnerable to temperature-associated damage. Thermotolerance levels caused by heat-shock protein Hsp70 are

initially low and have been shown to plateau at around twelve hours after embryogenesis (Feder et al. 1996), increasing survival at extreme temperatures (Welte et al. 1993). The absence of Hsp70 in earlier life stages may make them more susceptible to even low-intensity thermal stress, and thus cause poorer health and thermotolerance as adults. Previous studies have experimented on eggs (Klockmann et al. 2017), larvae (Roberts and Feder, 1999), pupae (Roberts et al. 2003), and adults (Stephanou et al. 1982) in response to acute thermal shock, but the effects of milder but longer-lasting chronic stress across development have remained to be fully investigated.

Over *D. melanogaster*'s ecological history as a human commensal, it has colonized a wide range of latitudes with varying thermal climates. Natural selection selects for traits that will perform better in each environment. As embryos, tropical populations of *D. melanogaster* have been found to survive more extreme temperature shocks compared to temperate populations (Lockwood et al. 2018). In contrast, the thermal tolerance of adults of tropical and temperate populations were no different when tested for upper thermal limits (Lockwood et al. 2018). Embryos are more immobile and thermally sensitive than adults, and therefore are more likely to experience adaptive variation in their upper thermal limits (Lockwood et al. 2018). Even though natural selection works on the fly's response to acute thermal stress as an embryo, whether a corresponding pattern of thermal adaptation across latitudes is found when chronic heat stress is applied at the embryonic stage is still unknown.

In this thesis, I tested how the impact of chronic thermal stress changes across development, and the extent to which natural selection has produced adaptive differences in thermotolerance between populations of *D. melanogaster* that originate from different thermal climates. In experiment 1, I asked whether flies develop greater thermal tolerance as they mature. Three different developmental stages of *D. melanogaster* were investigated, allowing me to pinpoint where along the developmental spectrum crucial steps in the development of thermotolerance occurred. Flies that were either 1, 24, or 60 hours old were placed into incubators that applied moderate and chronic heat or cold stress for the remainder of their development until they became adults. This allowed me to compare different developmental stages of fruit flies and assess the variation in thermotolerance and susceptibility of thermal stress to developmental processes between them. Thermal stress can affect many phenotypic

traits such as the proper growth of wings, and the ability to eclose into adults. To capture as many potential impacts as possible, I assayed survival and adult morphology, and measured thermal performance by assessing upper and lower thermal limits. These results provided indications for where the most sensitive steps of development occur. This project provided important insight for understanding how *D. melanogaster* responds to thermal stress applied during development.

In experiment 2, I asked whether populations living in chronically hotter environments showed evidence of evolved resistance to stress damage. Two temperate and two tropical populations were compared. These populations were placed into incubators when they were 1-hour old, which applied moderate and chronic heat and cold stress throughout the rest of development. The same thermal performance results and survival and adult morphology as described above were obtained for the tropical and temperate populations. This gave insight into how different populations of *D. melanogaster* have evolved to react to chronic thermal stress at the early developmental stage. The potential of *D. melanogaster* populations to evolve in response to changing average temperatures due to climate change is important to understand the migration, fitness, and survivability of *D. melanogaster* populations around the globe.

Materials & Methods:

Part 1: Timing of developmental stress:

Fly rearing and temperature manipulation

Thermal tolerance experiments were performed with Canton-S strain *Drosophila melanogaster* sourced from the Bloomington Drosophila Stock Center at Indiana University Bloomington. The stock flies were maintained in a 25°C incubator on a 12:12 h light cycle at 60% humidity. To collect eggs of known age, six vials with only growing larvae and no adults were set aside for one week, to ensure all the flies used were 0-7 days old. Flies were anesthetized with CO2, and enough flies to have ~150 mating pairs were placed in a cylindrical plastic mating vial with an agar dish containing yeast paste for food on one side and a mesh screen on the other side for adequate air circulation. Mating vials were placed in a 25°C

incubator for two days, with fresh yeast paste supplied every day. To collect embryos, a fresh agar plate was provided for 1 hour. For the 1-hour treatment, this agar plate was immediately collected and divided into six sections, with even numbers of embryos on each. For the 24- and 60-hour treatments, the fresh agar plate, after 1 hour, was separated from the mating flies and placed in the 25℃ incubator for 23 and 59 more hours, respectively. After that time had elapsed, the agar plates were divided into six sections as described above. To assess vulnerability to developmental heat and cold stress, each section was placed into a fresh vial, and the vials were evenly distributed across 18℃, 25℃ (control), and 30℃ incubators. Around 18-36 total vials were created for each developmental temperature, for each treatment. To ensure thermal stress was only being applied during development, adults were collected daily from all the vials once they eclosed and congregated in fresh vials in the 25℃ incubator.

Development and Eclosion success

To test whether cold or heat stress affected the ability of embryos to develop into pupae, the number of total pupae shells for each developmental temperature and treatment were recorded for each vial. To control for variation in the initial number of embryos laid by females, numbers at 18℃ and 30℃ were expressed as a proportion of the 25℃ control.

To test whether thermal stress affected the ability of flies to successfully eclose, the number of black (failed) and empty (successful) pupae shells were recorded for each vial, after enough time had passed for all the adults to eclose. Pupal shells were observed on the wall and food of the vials to determine the proportion of proper eclosion for each temperature for each treatment.

Critical thermal maximum (CTmax) and minimum (CTmin):

To determine tolerance of extreme heat, I determined the critical thermal maximum (CTmax), which was the temperature at which all major motor functioning was lost. Only the 1-hour and 24-hour transfer treatments were assayed for heat and cold tolerance. CTmax measurements were performed on 40 males and 40 females that developed in each temperature for each treatment, half of which were 2-day old flies and half were 7-day old flies. Eighteen flies at a time were inserted into individual 2 mL self-standing screw cap tubes. The tubes were attached to a wooden rod that was inserted into a clear, horizontal column with a concentric outer

jacket of circulating polycool liquid set to 25°C, with plugs on both sides of the tube to conserve the temperature of the circulating liquid and to hold the rod in place. The column was connected to a programmable water bath which increased the temperature of the circulating liquid by 0.25°C min^{-1}. A thermocouple temperature sensor was inserted directly into the center of the column to monitor the heat ramping experienced by the flies to the nearest 0.1°C. Starting at 35°C, flies were continuously checked for movement and the temperature at which a fly ceased to move was recorded.

To determine extreme cold tolerance for the treatments, I determined their critical thermal minimum (CTmin). Approximately one hundred flies were released into the same column as used for CTmax but oriented vertically, with the circulating liquid temperature set to 25°C. After five minutes, the temperature of the circulating water decreased by 0.25°C min^{-1}. Because healthy flies climbed to the top of the tube at the beginning of the experiment, at 12°C, I counted and removed flies that did not climb as an assay of climbing ability. Each 0.2°C increment along the path (Ex: 9.8-9.9°C) had a respective collecting tube. A funnel was inserted into the bottom of the vertical tube, allowing for collection tubes to be placed directly under the funnel. The number of flies that fell into each respective collecting tube represented the number of flies that experienced that temperature as their CTmin. Four CTmin runs were performed across each temperature from each treatment. Two of the CTmins were performed on 2-day old flies and two were performed on 7-day old flies.

Wing morphology

To test whether thermal stress affected the successful development of wings, I inspected all flies tested for CTmin for proper or deformed wings. Proper wings were large and had a consistent shape and look (Figure 1a). Deformed wings were crumpled, black, and were not spread out like wings typically are (Figure 1b).

(a)  (b) 

Figure 1. Images of Canton-S flies with proper (a) and deformed (b) wings.

Part 2: Temperate versus tropical fly populations:

Fly rearing and temperature manipulation

To test for adaptive divergence in developmental stress resistance, I compared two isogenic temperate strains and two isogenic tropical strains of *D. melanogaster*. The two temperate strains both originated from East Calais, VT (VT8 & VT10). The two tropical strains originated from Guam, USA (Guam) and Chiapas, Mexico (Chiapas). Although these were all isogenic lines and therefore not representative of the standing genetic variation at any site, they did represent a snapshot of the overall genetic pool represented across broad geographic areas around the globe. They have been in culture for approx. 10 years for the VT lines and approx. 15 years for the tropical lines (Lockwood et al. 2018). Geographic coordinates of collection locations are shown in Table 1.

Table 1. Collection site locations, regions, climate zones and maximum habitat temperatures of the warmest month of the year (Tmax) from 1950 to 2000 (WorldClim; Hijmans et al. 2005).

Collection locale	Lat. (°N)	Long. (°E)	Region	Climate zone	T_{max} (°C)
East Calais, Vermont, USA (VT8 & VT10)	44.4	-72.4	North America	North Temperate	25.7
Chiapas de Corzo, Chiapas, Mexico (Chiapas)	16.7	-93.0	Central America	Tropics	34.1
Guam, USA (Guam)	13.4	144.8	Oceania	Tropics	30.6

The stock flies were maintained in a 25℃ incubator on a 12:12 h light cycle at 60% humidity. Embryos were collected as described in part 1 above and were all transferred to temperature treatments after one hour. To capture the upper limit of performance for more heat-adapted populations, a 32℃-stress treatment incubator was added to include a temperature slightly beyond the upper limit of Canton-S. To ensure thermal stress was only being applied during development, adults were collected daily from all these vials, once they eclosed, and congregated in fresh vials at 25℃.

Total pupal production and the proportion of pupae that either successfully or failed to eclose were recorded as described above for each developmental temperature and population. The CTmax and CTmin were determined for each treatment, as described above. The CTmax of 20 males and 20 females was determined for each temperature for each population, utilizing only 2-day old adults. Two CTmin runs were performed for each temperature for each population, utilizing only 7-day old adults. From the flies tested for CTmin, the proportion of successful climbing and the proportion of proper wing development was recorded. Not enough adults successfully eclosed for Guam, VT8, and VT10 flies reared at 32℃ to adequately gather CTmax and CTmin data for these groups.

Statistical analyses:

Timing of developmental stress

Cumulative distribution plots in Graph Pad were used to create cumulative proportion curves for CTmin and CTmax data. JMP Pro 15 was used for all statistical analyses. For each phenotypic and performance measure, I used an ANOVA to determine the main effect of timing of egg transfer (life stage), developmental temperature, and their interaction. The proportion of eclosion, climbing, and deformed wing data were transformed, ($ArcSin(\sqrt{Prop})$, to normalize the data. Pairwise Tukey's HSD and Student's t-test (when only two groups being compared) post-hoc tests were performed to determine any statistical differences between groups.

Temperate versus tropical fly populations

To compare our tropical and temperate populations, two ANOVAs were used. The first ANOVA was used to determine the main effect of climate type (tropical or temperate), developmental temperature, the effect of sites nested within a climate type, and the interaction effect of climate type and developmental temperature. The second ANOVA was used to determine the main effect of developmental temperature, sites that came from different regions, and the interaction effect of site and developmental temperature. The proportion of eclosion, climbing, and deformed wing data were transformed, ($ArcSin(\sqrt{Prop})$, to normalize the data. Pairwise Tukey's HSD and Student's t-test (when only two groups being compared) post-hoc tests were performed to determine any pairwise statistical differences between groups.

Results:

Part 1: Timing of developmental stress

Development to the pupal stage at 18°C was more successful in later developmental stages than early stages. There was a significant effect of life stage on pupal development at 18°C (ANOVA, main effect of life stage, $F_{2, 213}$=5.506, P=0.0047). Compared to the control temperature, 24-hour flies were able to reach the pupal stage at 18°C at a higher proportion than 1-hour flies (Tukey HSD post-hoc test, P<0.05). Sixty-hour flies were able to reach the pupal stage at 18°C at the same proportion of 1- and 24-hour flies. The stage of development of fruit flies had no effect on the ability of flies to reach the pupal stage for flies reared at 30°C (ANOVA, main effect of life stage, $F_{2, 219}$=1.338, P=0.2646).

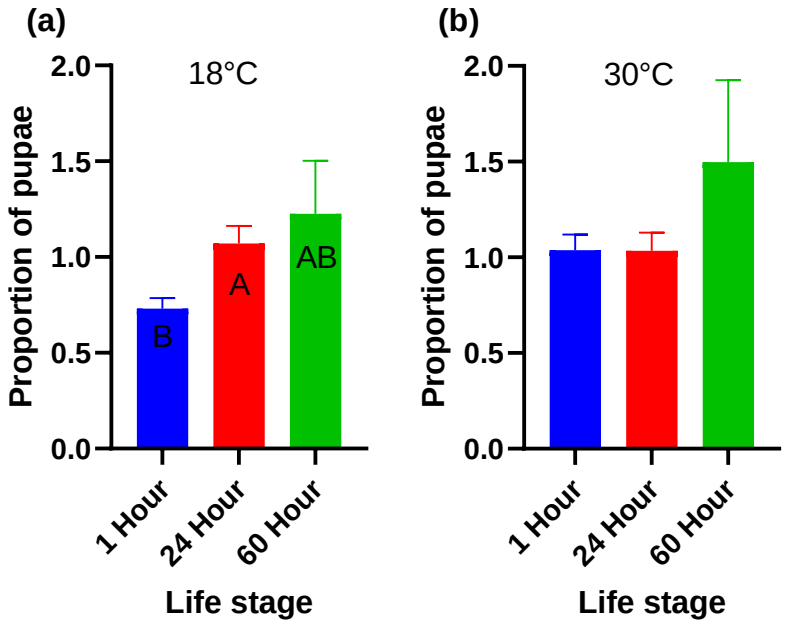


Figure 2. Proportion of pupae produced at low and high temperatures relative to the control. Proportion of flies that successfully developed to the pupal stage relative to the 25°C control, separated by life stage. Bars surrounding columns represent the standard error of the mean. (a) Proportion of flies across life stages that developed to pupae at 18°C relative to 25°C. (b) Proportion of flies across life stages that developed to pupae at 30°C relative to 25°C. Bars not connected by the same letter are significantly different, determined by a pairwise Tukey's HSD post-hoc test, with earlier letters indicating a higher mean value.

Eclosion success while experiencing cold and heat stress was greater for later developmental stages than early embryonic stages. Proportion eclosing differed significantly across life stages (ANOVA, main effect of life stage, $F_{2, 628}$=145.5, P<0.0001), with 24-hour flies eclosing at a higher proportion than 1-hour flies (Tukey HSD post-hoc test, P<0.05), but no difference in eclosion success from 24- to 60-hour flies. There was also a significant effect of development temperature on eclosion success (ANOVA, main effect of temperature, $F_{2, 628}$=108.2, P<0.0001). Flies reared at 25°C eclosed better than flies at 18°C, which eclosed greater than flies at 30°C (both P<0.05). There was a significant effect of developmental temperature across life stages (ANOVA, life stage x temperature interaction, $F_{4, 628}$= 64.88, P<0.0001). For 1-hour flies, 18°C flies eclosed at a lower proportion than 25°C flies (P<0.05),

with that gap disappearing in 24-hour flies. Flies reared at 30℃ similarly eclosed at a greater proportion in 24-hour flies than 1-hour flies ($P<0.05$), but by 60 hours the gap between 25℃ and 30℃ flies in eclosion success was gone.

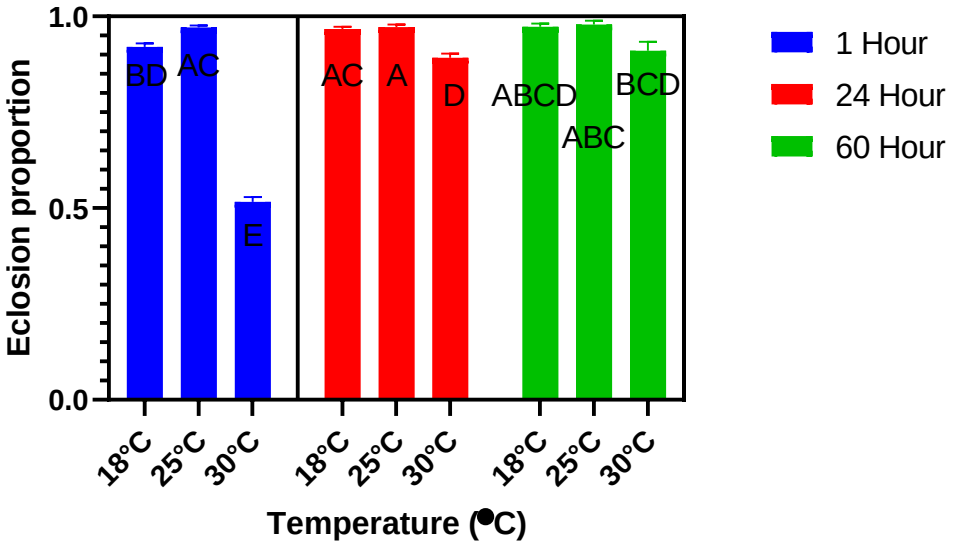


Figure 3. Proportion of eclosion differed by life stage and developmental temperature. Proportion of flies that successfully eclosed from pupae into adults, separated by life stage and developmental temperature. Bars surrounding columns represent the standard error of the mean. Proportion of eclosion for 18℃ flies recovered to control levels in 24-hour flies. Flies reared at 30°C eclosed better at 24 hours than 1 hour and recovered to control levels in 60-hour flies. Bars not connected by the same letter are significantly different, determined by a pairwise Tukey's HSD post-hoc test, with earlier letters indicating greater value.

Later developmental stages successfully climbed at a greater proportion than early developmental stages. There was a significant effect of life stage on proportion of climbing (ANOVA, main effect of life stage, $F_{1, 3167}=84.42$, $P<0.0001$). Twenty four-hour flies were able to climb to the top of the testing apparatus during CTmin testing at a higher proportion than 1-hour flies. There was a significant effect of development temperature on climbing proportion (ANOVA, main effect of temperature, $F_{2, 3167}=66.10$, $P<0.0001$). Flies reared at 30℃ climbed at a lower proportion than the control group, with 18℃ flies climbing at a greater proportion than the control (Tukey HSD post-hoc test, both $P<0.05$). The interaction effect of life stage and

developmental temperature had an impact on climbing proportion (ANOVA, life stage x temperature interaction, $F_{2, 3167}$=81.78, P<0.0001). For 1-hour flies, 18℃ flies climbed at a greater proportion than 25℃ flies and 30℃ flies climbed at a worse proportion than 25℃ flies (both P<0.05). These gaps in climbing ability between the developmental temperature groups at 1 hour disappeared for 24-hour flies, with all developmental temperatures climbing at the same proportion.

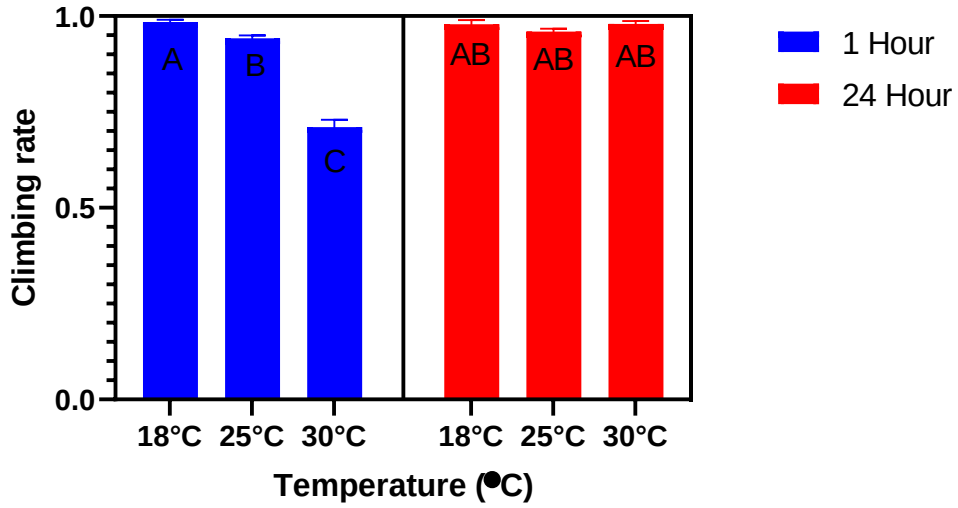


Figure 4. Proportion of climbing differed by life stage and developmental temperature. Proportion of flies that successfully climbed during CTmin assays, separated by life stage and developmental temperature. Bars surrounding columns represent the standard error of the mean. Differences in climbing proportion across developmental temperatures for 1-hour flies disappeared in 24-hour. Bars not connected by the same letter are significantly different, determined by a pairwise Tukey's HSD post-hoc test, with earlier letters indicating greater value.

Later developmental stages experienced a lower proportion of deformed wings when experiencing thermal stress than early developmental stages. One-hour flies developed abnormal wings at a greater proportion than 24-hour flies, with 15% of 1-hour flies developing deformed wings (ANOVA, main effect of life stage, $F_{1, 1171}$=17.79, P<0.0001). Proportion of deformed wings differed across developmental temperatures (ANOVA, main effect of temperature, $F_{2, 1171}$=69.91, P<0.0001). All flies reared at 18℃ and 25℃ had a negligible proportion of deformed wings, with 26.48% of all 30℃ flies developing deformed wings (Tukey HSD post-hoc test, both

P<0.05). The interaction of life stage and development temperature influenced the proportion of deformed wings (ANOVA, life stage x temperature interaction, $F_{2, 1171}$=24.77, P<0.0001). One-hour flies reared at 30℃ had a higher proportion of deformed wings than 1-hour flies reared at 25℃ and 18℃ (both P<0.05). Flies reared at 30℃ had a lower proportion of deformed wings for 24-hour flies than 1-hour flies (P<0.05). Twenty four-hour flies reared at 30℃ had the same proportion of deformed wings of 24-hour flies reared at 25℃ and 18℃.

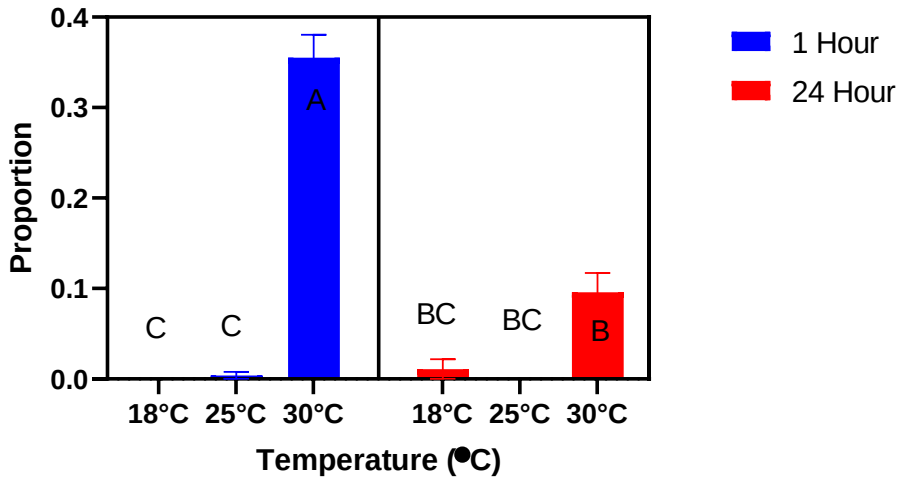


Figure 5. Proportion of deformed wings differed by life stage and developmental temperature. Proportion of flies that developed deformed wings, separated by life stage and developmental temperature. Bars surrounding columns represent the standard error of the mean. Proportion of deformed wings for 24-hour flies reared at 30℃ was smaller than 1-hour flies reared at 30℃ and was equal to 24-hour flies reared at 18℃ and 25℃. Bars not connected by the same letter are significantly different, determined by a pairwise Tukey's HSD post-hoc test, with earlier letters indicating greater value.

Later developmental stages were better acclimated to cold stress during development than early stages. Adult CTmin values were significantly lower in 24-hour flies than 1-hour flies. (ANOVA, main effect of life stage, $F_{1, 2779}$=71.02, P<0.0001). Flies reared at different developmental temperatures had different CTmin values (ANOVA, main effect of temperature, $F_{2, 2779}$=486.0, P<0.0001). CTmin values for all 18℃ flies were lower than the control, while all 30℃ flies had CTmin values greater than the control (Tukey HSD post-hoc test, both P<0.05).

The extent of this change differed by life stage (ANOVA, life stage x temperature interaction, $F_{2, 2779}$=50.40, P<0.0001). CTmin values for 25℃ and 30℃ flies were unaffected going from 1 to 24 hours, but 18℃ flies transferred at 24 hours were significantly lower for CTmin values than those transferred at 1 hour (P<0.05), with 24-hour flies experiencing cold stress having a further gap in performance from its respective control group than 1-hour flies that experienced cold stress (both P<0.05).

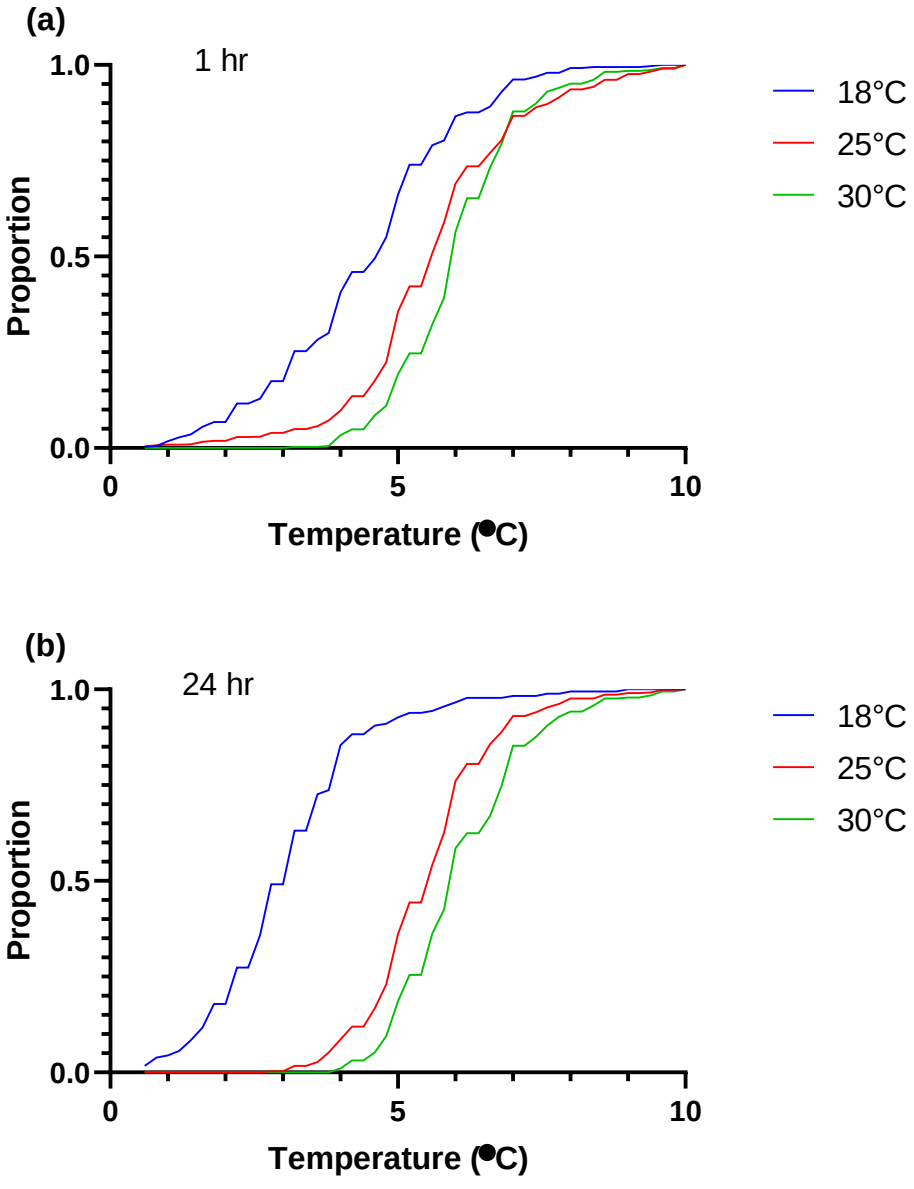


Figure 6. Cumulative critical thermal minimum (CTmin) curves across developmental temperatures for 1-hour (a) and 24-hour flies (b). (a) Proportion of 1-hour adults that retained motor control after extreme cold temperatures starting at 10°C (see Methods for rate of temperature change). (b) Proportion of 24-hour adults that retained motor control after extreme cold temperatures starting at 10°C. The cumulative proportion retaining motor control is displayed from right to left.

Later developmental stages experiencing heat stress had an improved upper thermal limit as adults compared to early stages experiencing heat stress. CTmax values were not different across life stages (ANOVA, main effect of life stage, $F_{1, 537}$=1.25, P=0.2641). CTmax values differed across developmental temperatures (ANOVA, main effect of temperature, $F_{2, 537}$=5.295, P=0.0053). Flies reared at 30℃ had the same CTmax as 18℃ and 25℃ flies, with 25℃ flies having a greater CTmax than 18℃ flies (Tukey HSD post-hoc test, P<0.05). The interaction effect of life stage and developmental temperature influenced CTmax values (ANOVA, life stage x temperature interaction, $F_{2, 537}$=4.815, P=0.0085). Flies reared at 18℃ had the same CTmax as 25℃ flies at 1 hour and 24 hours. For 1-hour flies, flies reared at 30℃ had lower CTmax values than 25℃ flies (P<0.05), but for 24-hour flies, flies reared at 25℃ and 30℃ had the same CTmax.

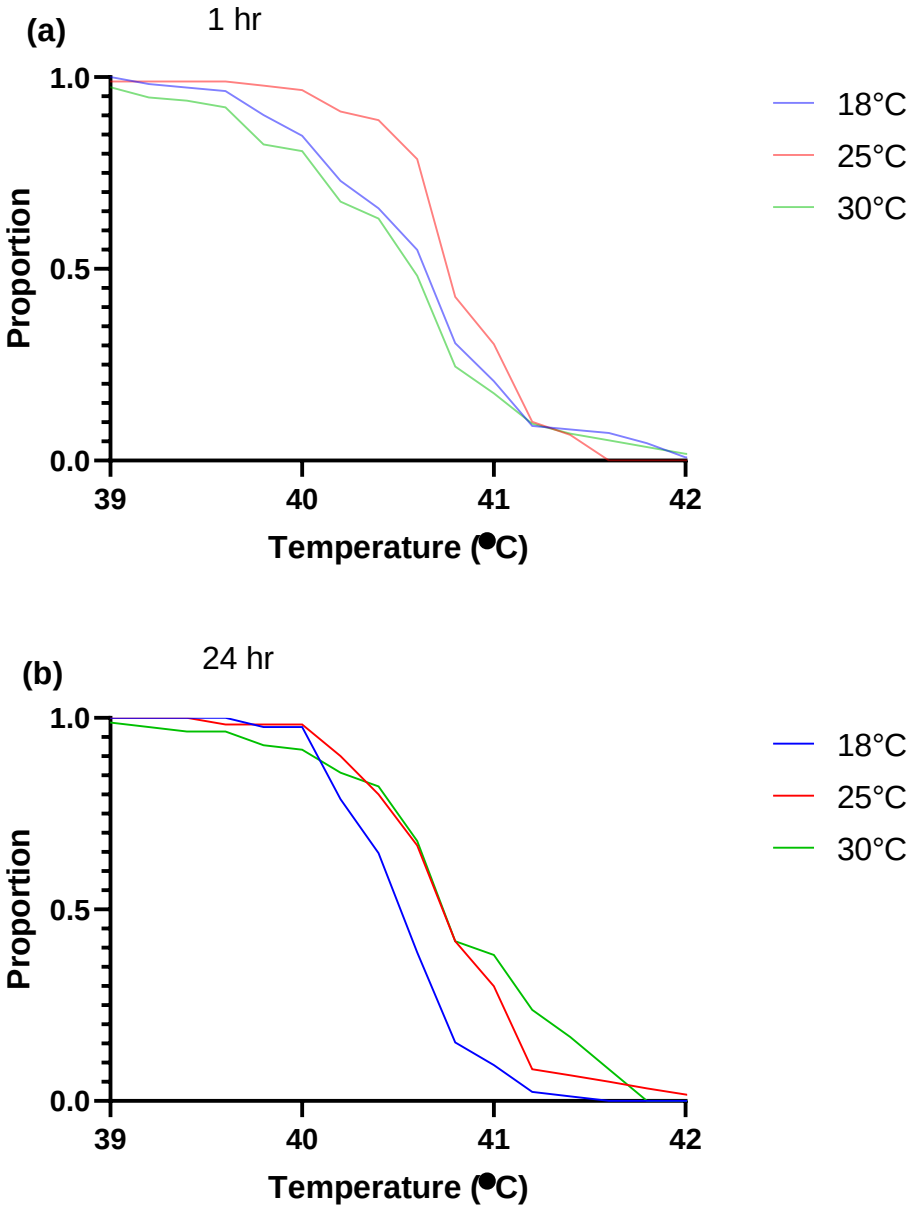
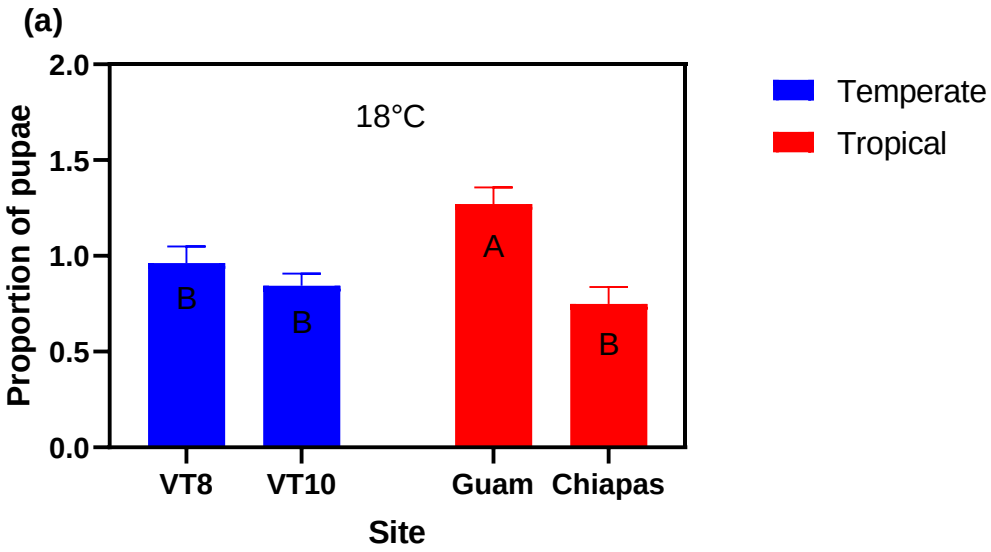


Figure 7. Cumulative critical thermal maximum (CTmax) curves across developmental temperatures for 1-hour (a) and 24-hour flies (b). (a) Proportion of 1-hour adults that retained motor control after extreme heat temperatures starting at 39°C (see Methods for rate of temperature change). (b) Proportion of 24-hour adults that retained motor control after extreme heat temperatures starting at 39°C.

Part 2: Temperate versus tropical fly populations:

Tropical populations were more likely to diverge in success of pupae development between each other than temperate populations were. The ability to reach the pupal stage at 18°C was the same for tropical and temperate flies (ANOVA, main effect of type, $F_{1, 176}$=1.233, P=0.2683). There was an effect of site nested within climate type on reaching the pupal stage at 18°C (ANOVA, main effect of site nested within type, $F_{2, 176}$=8.632, P=0.0003). Guam flies reached the pupal stage at 18°C at a higher proportion than Chiapas flies (Tukey HSD post-hoc test, P<0.05), with both Vermont populations able to reach the pupal stage at 18°C at the same rate. Tropical flies reached the pupal stage at 30°C by a greater amount than temperate flies (ANOVA, main effect of type, $F_{1, 213}$=12.12, P=0.0006). There was an effect of site nested within climate type on reaching the pupal stage at 30°C, but neither of the pairwise comparisons were significant (ANOVA, main effect of site nested within type, $F_{2, 213}$=3.254, P=0.0405). The ability to reach the pupal stage at 32°C was the same for tropical and temperate flies (ANOVA, main effect of type, $F_{1, 69}$=0.0664, P=0.7973). There was an effect of climate type nested sites on reaching the pupal stage at 32°C (ANOVA, main effect of site nested within type, $F_{2, 69}$=9.669, P=0.0002). Guam flies reached the pupal stage at 32°C at a higher proportion than Chiapas flies (P<0.05), with both Vermont populations able to reach the pupal stage at 32°C at the same rate.



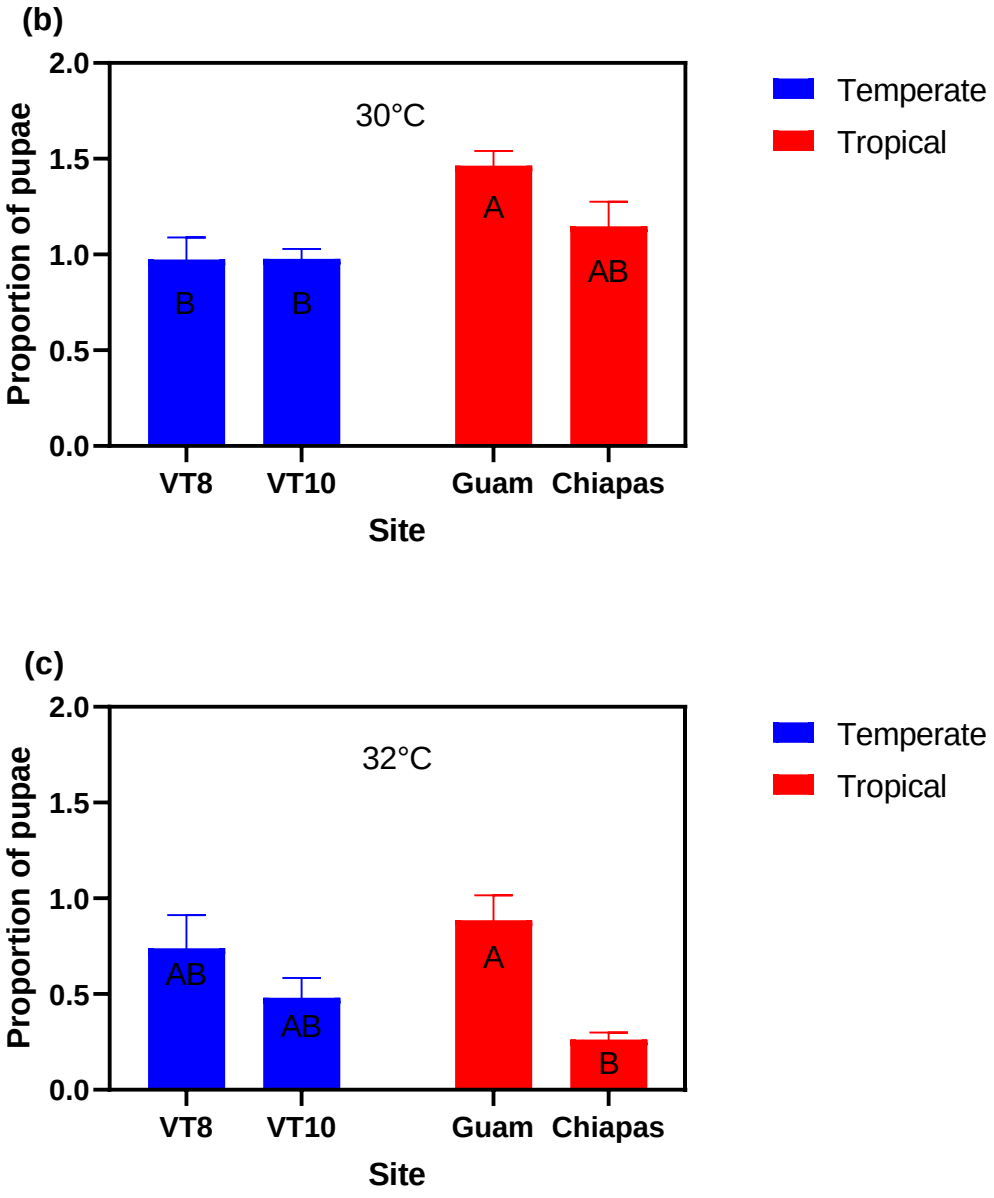


Figure 8. Proportion of pupae produced at low and high temperatures relative to the control. Proportion of flies that successfully developed to the pupal stage relative to the control, separated by climate type and site. Bars surrounding columns represent the standard error of the mean. (a) Proportion of flies across sites nested within climate type that developed to pupae at 18℃ relative to 25℃. (b) Proportion of flies across sites nested within climate type that developed to pupae at 30℃ relative to 25℃. (c) Proportion of flies across sites nested within climate type that developed to pupae at 32℃ relative to 25℃. Bars not connected by the same letter are significantly different, determined by a pairwise Tukey's HSD post-hoc test, with earlier letters indicating greater value.

Tropical flies had improved eclosion success at 32℃ than temperate flies. Tropical flies eclosed at a higher proportion than temperate flies (ANOVA, main effect of type, $F_{1, 733}$=40.10, P<0.0001). Eclosion success differed between developmental temperatures (ANOVA, main effect of temperature, $F_{3, 733}$=571.0, P<0.0001). All the extreme developmental temperatures eclosed at a lower proportion than the control group, with 18℃ flies eclosing at a significantly higher proportion than 30℃ flies, and 30℃ flies eclosing better than 32℃ flies (Tukey HSD post-hoc test, all P<0.05). The interaction effect of climate type and developmental temperature was significant on the proportion of eclosion (ANOVA, type x temperature interaction, $F_{3, 733}$=12.01, P<0.0001). Temperate flies reared at 18℃ eclosed worse than 25℃ temperate flies (P<0.05). Flies reared at 30℃ were not different across the climate types, with tropical flies eclosing at a greater proportion at 32℃ than temperate flies (P<0.05).

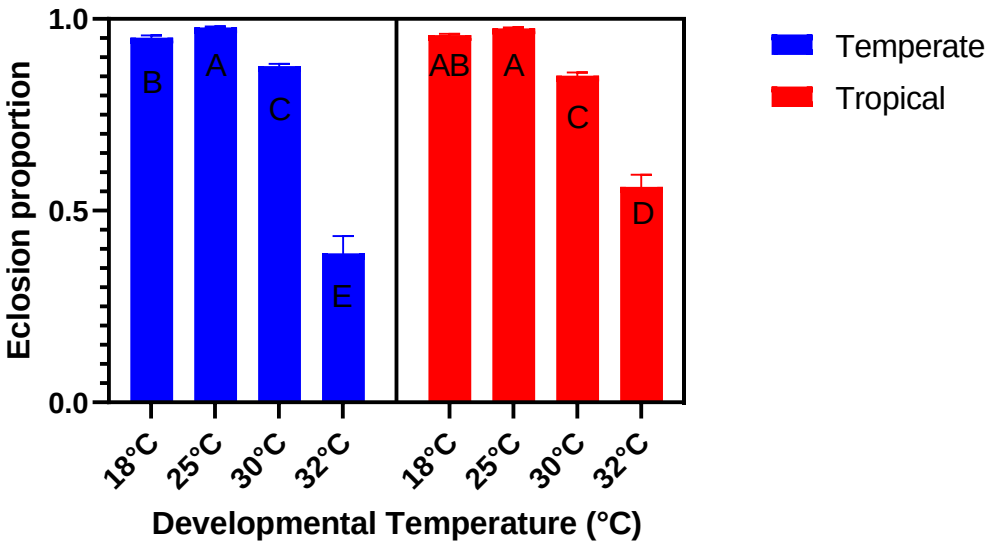


Figure 9. Proportion of eclosion differed by life stage and developmental temperature. Proportion of flies that successfully eclosed from pupae into adults, separated by climate type and developmental temperatures. Bars surrounding columns represent the standard error of the mean. The gap between 32℃ and 25℃ flies in eclosion success for tropical flies was less than temperate flies. Bars not connected by the same letter are significantly different, determined by a pairwise Tukey's HSD post hoc-test, with earlier letters indicating greater value.

There were differences between sites of the same climate type for eclosion success (ANOVA, main effect of site nested within type, $F_{2, 733}$=97.13, P<0.0001). Chiapas flies eclosed at a greater proportion than Guam flies (Tukey HSD post-hoc test, P<0.05), with VT8 and VT10 flies eclosing at the same proportion. There were also differences between sites of different climate types (ANOVA, main effect of site, $F_{3, 727}$=121.3, P<0.0001). When results were reanalyzed without region of origin as a factor, Chiapas flies eclosed better than all the other sites (all P<0.05), with VT10 flies eclosing better Guam flies (P<0.05) and VT8 flies eclosing at the same proportion as Guam flies. The interaction effect of sites and developmental temperature was significant (ANOVA, site x temperature interaction, $F_{9, 727}$=28.92, P<0.0001). Chiapas flies reared at 30°C and 32°C eclosed better than 30°C and 32°C flies for all other sites, respectively (all P<0.05). Guam flies reared at 30°C eclosed the same as 30°C VT8 flies and worse than 30°C VT10 flies (P<0.05). Guam flies reared at 32°C had the same proportion of eclosion as 32°C VT8 and VT10 flies.

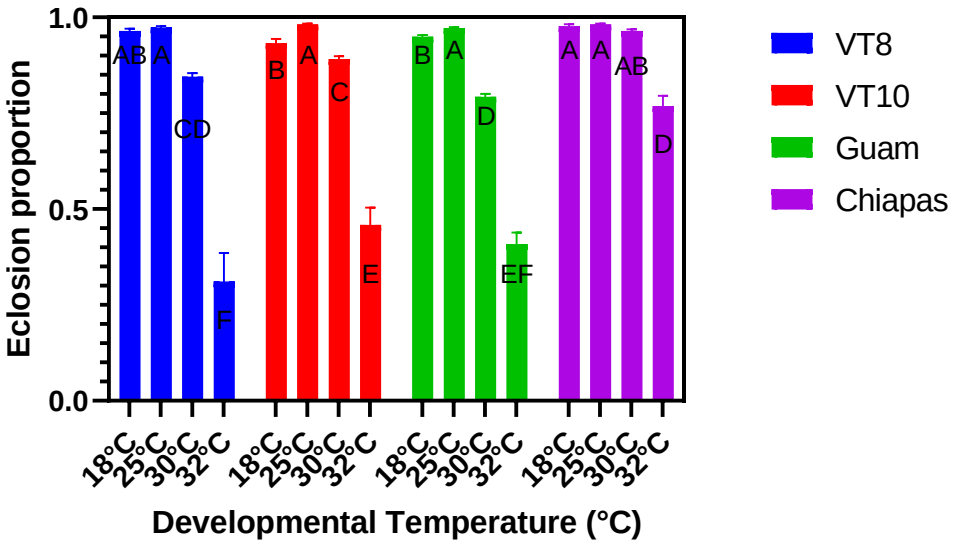


Figure 10. Proportion of eclosion differed by site and developmental temperatures. Proportion of flies that successfully eclosed from pupae into adults, separated by site and developmental temperatures. Bars surrounding columns represent the standard error of the mean. Chiapas flies eclosed better at 30°C and 32°C than the other sites, with Guam, VT8, and VT10 flies eclosing similarly at all developmental temperatures. Bars not connected by the same letter are significantly different, determined by a pairwise Tukey's HSD post-hoc test, with earlier letters indicating greater value.

The proportion of climbing was also different across sites and developmental temperatures. Temperate flies had a greater proportion of climbing than tropical flies (ANOVA, main effect of type, $F_{1, 2734}$=23.64, P<0.0001). The effect of developmental temperature on climbing proportion was significant (ANOVA, main effect of temperature, $F_{2, 2734}$=13.97, P<0.0001). Flies reared at 25℃ and 30℃ had the same proportion of climbing, with 18℃ flies being greater than both groups (Tukey HSD post-hoc test, both P<0.05). Proportion of flies that climbed differed for sites within climate types (ANOVA, main effect of site nested within type, $F_{2, 2734}$=56.52, P<0.0001). Chiapas flies climbed better than Guam flies, and VT10 flies climbed better than VT8 flies (both P<0.05). Sites from different climate types also differed for climbing proportion (ANOVA, main effect of site, $F_{2, 2731}$=73.07, P<0.0001). VT10 and Chiapas flies climbed at the same proportion, with Guam flies climbing at a worse proportion than both of those sites (both P<0.05) and VT8 flies climbing worse than all other sites (all P<0.05). There was no interaction effect between climate type and developmental temperature on the proportion of climbing (ANOVA, type x temperature interaction, $F_{2, 2734}$=0.4893, P=0.6131), but sites differed in the effect of developmental temperatures on the proportion of climbing (ANOVA, site x temperature interaction, $F_{5, 2731}$=14.49, P<0.0001). Chiapas, VT8, and VT10 flies had the same respective proportion of climbing at 18°C and 25°C. VT10 flies reared at 30°C climbed better than 30°C Chiapas flies (P<0.05). Guam and VT8 flies reared at 30°C flies had the same proportion of climbing, both climbing worse than 30°C Chiapas flies (both P<0.05).

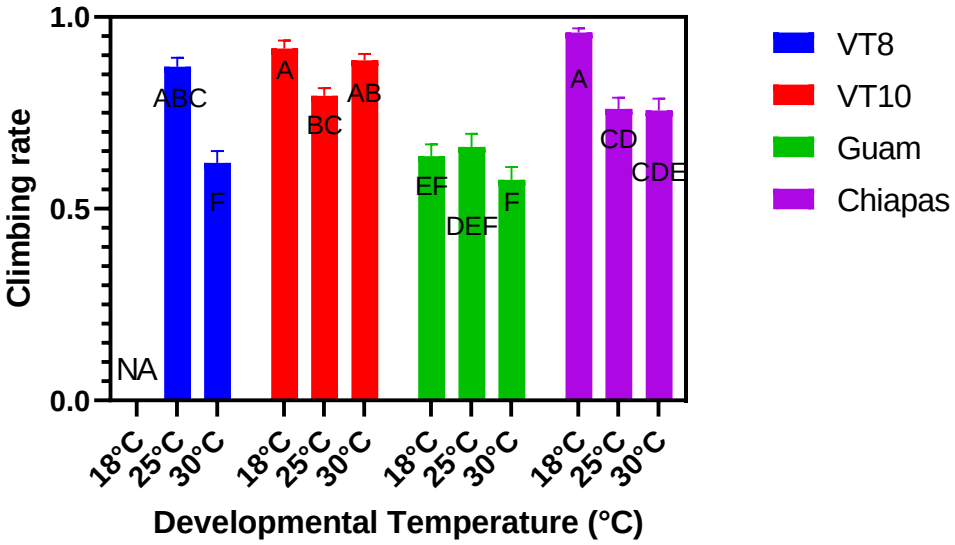


Figure 11. Proportion of climbing differed by site and developmental temperature. Proportion of flies that successfully climbed during CTmin assays, separated by site and developmental temperatures. Bars surrounding columns represent the standard error of the mean. VT10 and Chiapas flies climbed better than Guam flies at all developmental temperatures. VT8 flies reared at 30°C climbed at the same proportion as 30°C Guam flies and worse than 30°C Chiapas and VT10 flies. Bars not connected by the same letter are significantly different, determined by a pairwise Tukey's HSD post-hoc test, with earlier letters indicating greater value.

Few deformed wings were identified in any of the populations. The proportion of deformed wings found were unaffected by climate type, developmental temperature, nested site, or developmental temperature interaction with climate type (ANOVA, main effect of type, $F_{1, 1356}$=0.0126, P=0.9105, main effect of temperature, $F_{2, 1349}$=1.303, P=0.2721, main effect of site nested within type, $F_{2, 1349}$=0.7997, P=0.4497, type x temperature interaction, $F_{2, 1349}$=1.141, P=0.3197). When results were reanalyzed without region of origin as a factor, interaction effect of site with developmental temperature also had no effect on proportion of deformed wings (ANOVA, site x temperature interaction, $F_{6, 1345}$=0.8150, P=0.5582).

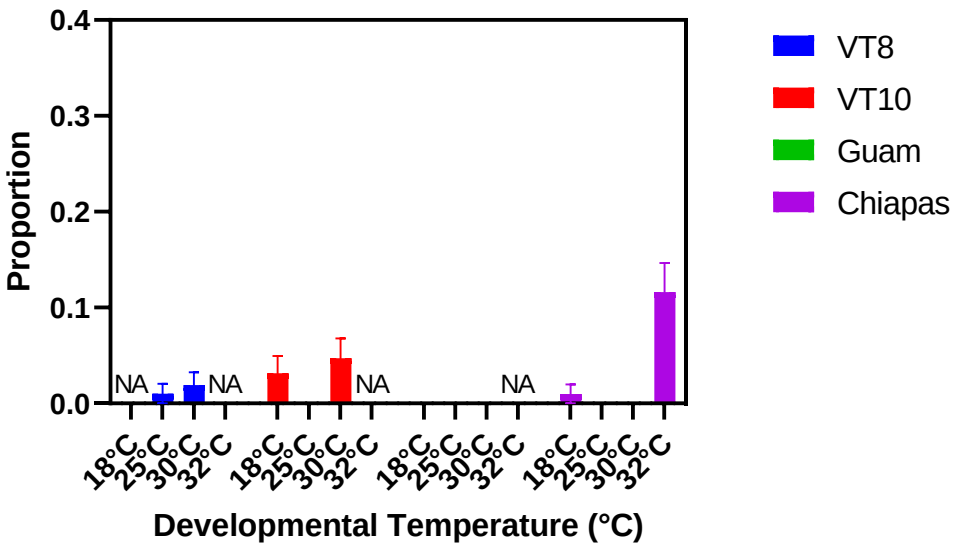


Figure 12. Proportion of deformed wings were the same across site and developmental temperature. Proportion of flies that developed deformed wings, separated by site and developmental temperatures. Bars surrounding columns represent the standard error of the mean. The interaction effect of site and developmental temperature had no effect on the proportion of deformed wings. Bars not connected by the same letter are significantly different, determined by a pairwise Tukey's HSD post-hoc test, with earlier letters indicating greater value. Sample sizes for VT8: 18°C (n=0 flies), 25°C (n=209), 30°C (n=239), 32°C (n=0). Sample sizes for VT10: 18°C (n=170), 25°C (n=233), 30°C (n=256), 32°C (n=0). Sample sizes for Guam: 18°C (n=234), 25°C (n=192), 30°C (n=231), 32°C (n=0). Sample sizes for Chiapas: 18°C (n=227), 25°C (n=213), 30°C (n=193), 32°C (n=249).

Lower thermal limits were consistent between developmental temperatures of different sites. Temperate and tropical flies did not differ for CTmin values (ANOVA, main effect of type, $F_{1, 2017}$=1.278, P=0.2584). Developmental temperature did influence CTmin values (ANOVA, main effect of temperature, $F_{2, 2017}$=138.2, P<0.0001). Flies reared at 18°C had a lower CTmin than 25°C flies, which was lower than 30°C flies (Tukey HSD post-hoc test, both P<0.05). Sites within climate types differed for CTmin values (ANOVA, main effect of site nested within type, $F_{2, 2017}$=13.49, P<0.0001). Within climate types, Guam flies had a lower CTmin than Chiapas flies and VT8 flies had a lower CTmin than VT10 flies (both P<0.05). Sites outside of climate types also differed for CTmin values (ANOVA, main effect of site, $F_{2, 2014}$=11.22, P<0.0001). Between sites, VT8 flies had the lowest CTmin and VT10 and Chiapas flies had the highest CTmin values (all P<0.05). Chiapas flies had the same CTmin as VT10 flies, and Guam flies had

the same CTmin as Chiapas flies but not VT10 flies ($P<0.05$). There was an interaction effect of site and developmental temperature on CTmin values (ANOVA, main effect of site x temperature interaction, $F_{5, 2014}=4.669$, $P=0.0003$). CTmin values for 18℃, 25℃, and 30℃ flies were respectively the same for all sites, except for 25℃ VT10 flies which had the same CTmin as 25℃ Chiapas flies but greater CTmin values than 25℃ Guam and VT8 flies (both $P<0.05$). The interaction effect of climate type and developmental temperature also influenced CTmin values (ANOVA, type x temperature interaction, $F_{2, 2017}=8.256$, $P=0.0003$). CTmin values for 18℃ and 30℃ flies were respectively the same for tropical and temperate flies. CTmin values for tropical flies reared at 25℃ were lower than 25℃ CTmin values for temperate flies ($P<0.05$), with 25℃ and 30℃ CTmin values for temperate flies being equal.

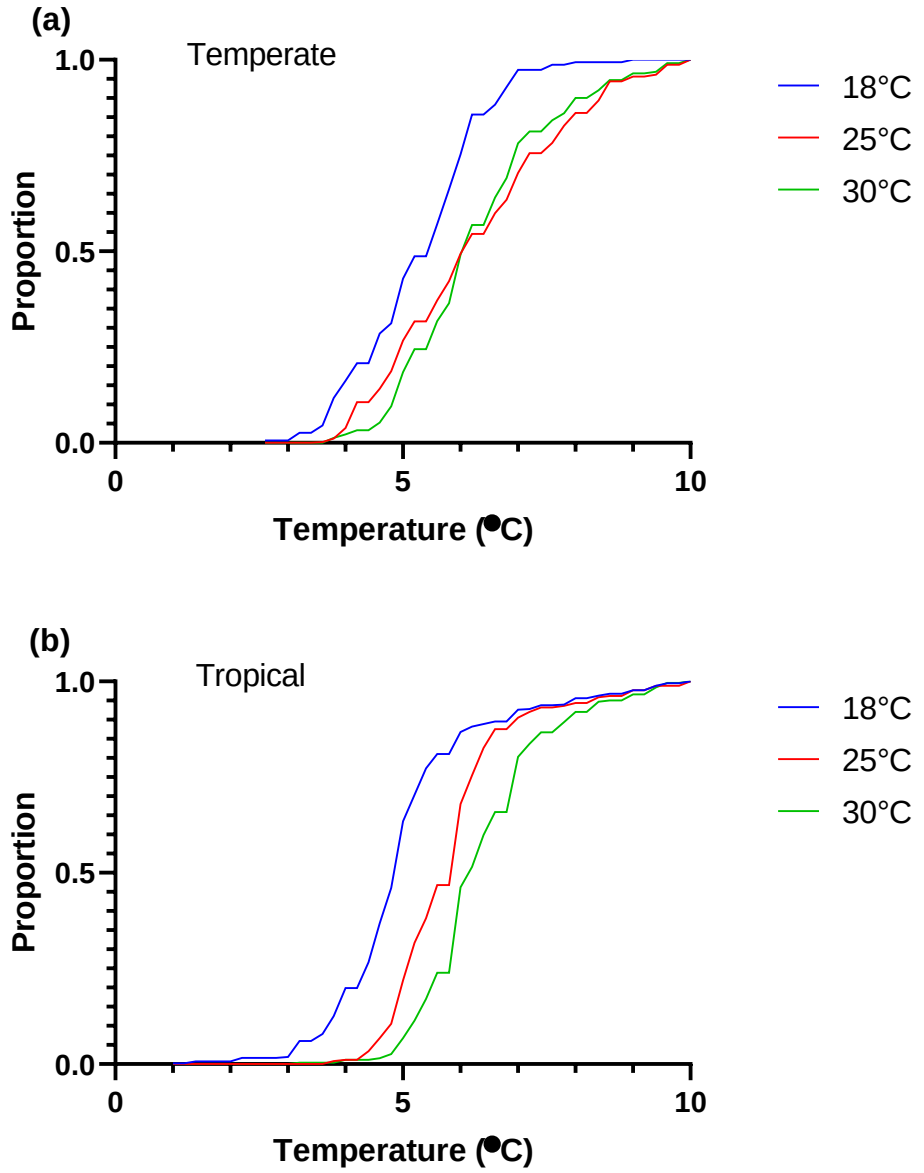


Figure 13. Cumulative critical thermal minimum (CTmin) curves across climate types for temperate (a) and tropical flies (b). (a) Proportion of temperate adults that retained motor control after extreme cold temperatures starting at 10°C (see Methods for rate of temperature change). (b) Proportion of tropical adults that retained motor control after extreme cold temperatures starting at 10°C. The cumulative proportion retaining motor control is displayed from right to left.

Chiapas flies exhibited greater upper thermal limits for flies reared at high temperatures. Temperate flies had a lower CTmax than tropical flies (ANOVA, main effect of type, $F_{1,}$

$_{1259}$=44.95, P<0.0001). CTmax values differed between developmental temperatures (ANOVA, main effect of temperature, $F_{2, 1259}$=138.2, P<0.0001). Flies reared at 30℃ had higher CTmax values than 25℃ flies, which had higher CTmax values than 18℃ flies (Tukey HSD post-hoc test, both P<0.05). Sites differed for CTmax values (ANOVA, main effect of site, $F_{3, 1255}$=43.60, P<0.0001). Guam, VT8, and VT10 flies all had the same CTmax values, with Chiapas flies having greater CTmax values than all the other sites (all P<0.05). The interaction effect of climate type and developmental temperature had no effect on CTmax (ANOVA, type x temperature interaction, $F_{2, 1259}$=2.860, P=0.0577). Interaction effect of site and developmental temperature differed for upper thermal limits (ANOVA, site x temperature interaction, $F_{6, 1255}$=11.33, P<0.0001). Guam, VT8, and VT10 30℃ flies had the same CTmax values, with 30℃ Chiapas flies having greater CTmax values than the rest of the sites (all P<0.05). Guam, Chiapas, and VT10 25℃ flies had the same CTmax values, with 25℃ VT8 flies having smaller CTmax values than the rest of the sites (all P<0.05).

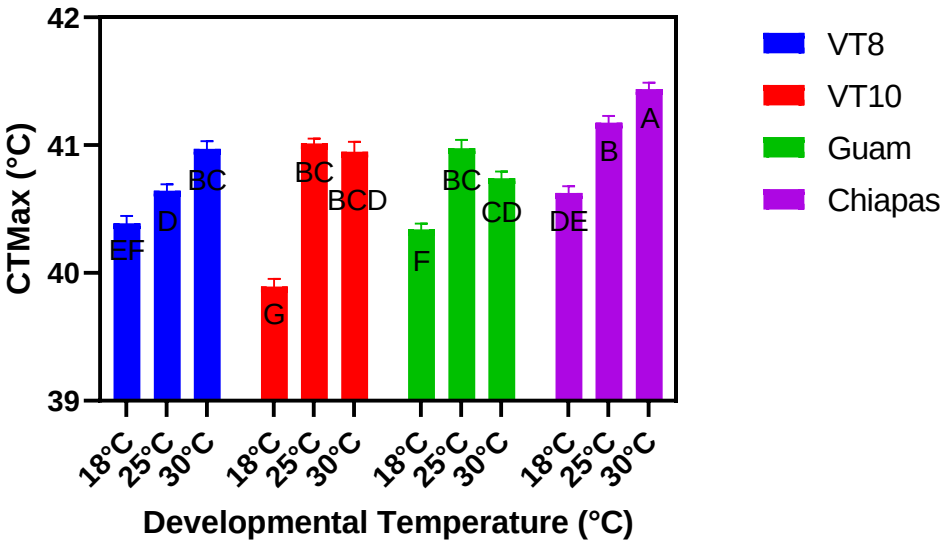


Figure 14. Critical thermal maximum (CTmax) differed across sites and developmental temperatures. Average CTmax, separated by site and developmental temperatures. Bars surrounding columns represent the standard error of the mean. Of 30℃ flies, Chiapas had the greatest CTmax values. Bars not connected by the same letter are significantly different, determined by a pairwise Tukey's HSD post-hoc test, with earlier letters indicating greater value.

Discussion:

After a fly is born it must undergo a series of crucial steps throughout development to eclose into a fully formed and healthy adult. Because thermal resistance develops over time in flies, along with many crucial steps of developing shortly after an embryo is born, my first question centered around if later developmental stages had increased resistance to thermal stress than early stages. If earlier developmental stages were more thermally sensitive, they should show greater developmental defects and experience inferior upper and lower thermal limits from moderate thermal stress compared to later developmental stages. Due to early developmental stages of fruit flies being more susceptible to thermal stress, our second question was centered around if thermal adaptation took place during development against thermal stress. It was hypothesized that thermal adaptation has led to adaptive divergence between tropical and temperate populations, with tropical and temperate populations exhibiting increased resistance against hot and cold stress during development, respectively.

Timing of developmental stress

Later developmental stages consistently demonstrated increased thermal resistance against moderate and chronic thermal stress than early stages. Twenty four-hour flies experienced a higher proportion of eclosion, pupae development at 18°C, climbing, and proper wing development. They also exhibited improved acclimation in CTmin and a complete rebound of performance of CTmax to control levels. Across the various phenotypic tests and observations, a persistent trend of greater resistance and acclimation at the 24-hour stage compared to the 1-hour stage showcases how *D. melanogaster* develops molecular mechanisms to resist thermal stress throughout development.

This result matches well with what is known about the development of the heat shock response, which is well developed by the 12-hour mark in development, with the accumulation of HSPs mostly complete by this stage (Welte et al. 1993). Extra copies of Hsp70 during all the larval instars induced greater expression of Hsp70 and had improved thermotolerance, during development, in the face of heat shock (Feder et al. 1996). Even though the flies in (Feder et al. 1996) were assessed for upper thermal limit during development instead of adulthood, their results show how increased Hsp70 expression can increase thermal tolerance at the upper

thermal limit of flies and mitigate damage induced by thermal stress. *Drosophila melanogaster* depends more heavily upon Hsp70 for thermotolerance than most organisms (Feder et al. 1996). The importance of HSPs against thermal stress along with the sufficient accumulation of HSPs by the 12-hour mark indicate that the 24-hour flies have already well-established buildup of HSPs and basal thermotolerance, giving it an advantage for stress-mitigation over earlier developmental stages. This advantage later in development, in addition to excising stress-damaged proteins, also promotes thermal resistance by limiting the production and buildup of harmful reactive oxygen species as well as limiting the accumulation of other intracellular toxic chemicals that could disrupt basic cellular functions (Roberts and Feder, 1999).

Thermal stress during development can disrupt the mechanisms that are needed for flies to develop into healthy pupae. Before flies could reach adulthood, 1-hour flies were less able to develop from eggs into pupae, with 24-hour flies being able to develop into pupae greater than 1-hour flies by up to 57% (Figure 2). The ability to develop into pupae during heat stress was not affected by life stage (Figure 2b). For cold stress, however, there was a change across life stages. Later developmental stages were less impacted by the cold stress and were better able to develop into pupae, showing how cold stress had the larger impact at the larval stage than heat stress (Figure 2a). By 24-hours, flies have fully developed their defense mechanisms for cold stress's effect on pupae development. Cold stress during early development can lead to the partial or complete denaturation of proteins, with this disruption influencing the fly's ability to develop into the pupae stage (Koštál et al. 2019). The massive upregulation of HSPs is an important defense mechanism against cold stress (Koštál et al. 2019). The reduced accumulation and expression of HSPs of 1-hour flies, compared to later developmental stages, may reduce their ability to react to cold stress and result in increased mortality leading up to the pupae stage (Feder et al. 1996). Heat stress at 30°C may be too mild to cause the necessary amount of denaturation during the larval stage to prevent the development of the fly into the pupal stage.

Even though heat stress does not affect survival to the pupal stage, early embryos clearly experience developmental damage that has cascading effects later, resulting in more severe disadvantages during the pupal stage affecting their ability to fully develop into the final adult stage. There are many steps that occur throughout development for a fruit fly to develop from an egg into a pupae. To complete its development and form a mature adult, flies must escape from

their pupal shells into the outside world. A failure to escape from their pupal shells is an indication of incomplete development somewhere along the pre-adult life stages. One-hour flies were severely affected by both cold and heat stress during development, with both the 18°C and 30°C groups for 1-hour flies eclosing at a lower proportion than the 25°C flies, especially for the 30°C group (Figure 3). For flies that did not experience thermal stress until 24 hours, the deleterious effect cold stress had on the 1-hour flies reared at 18°C flies were no longer present, and the effect of heat stress on the 30°C group was reduced (Figure 3). By the 60-hour mark during development, cold and heat stress had no effect on the ability of flies to properly eclose into adults (Figure 3). Thermal stress can be very deleterious to flies during development. In addition to affecting the development of the fly, thermal stress can affect critical cellular functions and, during larvae and pupae development, decrease the volume of conserved parts of the brain (Roberts et al. 2003). Therefore, the accumulation of stress-damaged proteins, altered structure of the brain, and affected critical cellular functions could have influenced the inferior eclosion success of the 1-hour flies at 18°C and 30°C, due to the reduced thermotolerance and HSPs levels at this life stage (Roberts et al. 2003). Of the early developmental flies that failed to eclose due to heat stress, up to 41% of them partially escaped from their pupae shells, indicating that heat stress impacted the ability of flies to escape from their pupae shells in addition to impacting developmental processes (Figure 3). Eclosion success could provide some insight into the timetable of the development of HSPs, as the heat stress defense mechanisms in flies could be partially developed by 24 hours and fully developed by 60 hours.

Wing formation primarily occurs during the pupal stage and fully expand in adults, with thermal stress impacting this development. Cold stress during both life stages did not affect the development of wings, indicating that the cold stress experienced at 18°C was not sufficient to disrupt the mechanisms behind wing development (Figure 5). One-hour flies experienced a high proportion of deformed wings after experiencing heat stress (Figure 5). By 24 hours, the rate of deformed wings decreased so much that heat stress no longer caused more deformed wings than flies reared at 25°C (Figure 5).

Several important steps occur in wing development around 24 hours in the early larvae stages. The wing field dictating where the wing will form is defined and the polarity along the dorso-ventral axis is established, all directed by the actions of important transcription factors

(TFs) such as wg, vg, and Ap, which become highly expressed around this stage, which signifies the onset of the second larvae instar (Diaz-Benjumea and Cohen, 1993; Grimm and Pflugfelder, 1996; Klein, 2001). Due to the buildup of these TFs at or around the 24-hour stage of development, already initiating important steps in wing development, wing development by this time could be sufficiently protected and complete to not be affected by the stress felt at 30°C. Flies during adulthood rely heavily on their wings to perform daily tasks and contribute to their fitness and survival. Flies require wings to be able to evade predators, find mates, and find shelter and food (Diaz-Benjumea and Cohen, 1993). A high proportion of deformed wings in a natural population experiencing chronic heat stress could prove very harmful to the success and survival of the population. Important developmental steps for healthy adult development are still being initiated at the 1-hour mark (Klein, 2001). Coupled with the fact that basal thermotolerance levels are still expanding, the risk posed by flies on their choice of egg-laying location is real.

Defects produced during metamorphosis due to thermal stress also appeared to impair locomotion. Heat stress during the first hour of development negatively impacted those flies' abilities as adults to climb the CTmin apparatus (Figure 4). By 24 hours, thermal stress no longer affected flies' abilities to climb the apparatus, having developed sufficient resistance to thermal stress on the development of locomotion (Figure 4). As discussed earlier, thermal stress during development could decrease the volume and functioning of the brain (Roberts et al. 2003). Thermal stress during development is known to affect learning and behavioral functions of the brain as well as the regulation of walking behaviors (Roberts et al. 2003). This, coupled with thermal stress having been shown to affect the development of wings and body segments, could contribute to 1-hour flies being limited in their abilities to climb the apparatus (Roberts and Feder, 1999). Walking and wings are important for the fitness and survival of flies, as they are involved in flight, courtship, territorial defense, and resource gathering (Robert and Feder, 1999).

The cumulative effects of early sublethal stress across all the earlier developmental problems are substantial in the development of fully formed, healthy adults. Flies at earlier developmental stages have been shown to be less successful in reaching the pupae stage at 18°C and less successful in reaching the adult stage at 18°C and 30°C. And once they reach adulthood, the 1-hour flies that experienced heat stress experienced larger chances of developing deformed wings as well as larger chances of experiencing locomotion disruptions. Non-lethal stressors

such as the 18°C and 30°C incubators, therefore, could have a large impact on natural populations as they will experience increased mortality at the pupae and adult stage and decreased performance, fitness, and survival due to the wing and locomotion deficiencies.

In addition to defects caused by thermal stress, potential benefits could arise such as acclimation to the abnormal temperatures that flies were reared in. However, at the early embryonic stage, the thermal stress inflicted overwhelms the possibility of thermal stress leading to an acclimation response. Moderate and chronic cold stress during development, as well as cold stress during adulthood, have been shown to lead to cold acclimation and reduce mortality at low temperatures and decrease lower thermal limit in adulthood (Colinet and Hoffmann, 2012). Starting at 1 hour, applying cold stress at 18°C positively prepared the flies as adults to respond to cold temperatures, giving them an advantage over flies that did not experience cold stress (Figure 6a). By 24 hours, the application of cold stress further acclimated flies to be resistant to abnormally cold temperatures, with 24-hour 18°C flies surviving to much colder temperatures than 18°C flies at 1 hour (Figure 6b). This is evidence that later development stages of fruit flies are better equipped to acclimate to sublethal cold stress than earlier developmental stages. The expression of Hsp70 as well as proteins that are expressed with Hsp70 such as Hsp40, Stv, and Fst have been shown to be upregulated in flies that experienced chronic cold stress during development (Colinet and Hoffmann, 2012). The upregulation of stress defense genes during developmental cold stress can lead to improved thermal tolerance for low temperatures during adulthood, as shown here.

Just like with 30°C flies measured for CTmin, 18°C flies showcased a reduced capability to survive at hot temperatures (Figure 7). This reduced heat resistance was not altered during the later developmental stage (Figure 7b). Once heat stress was applied during the 1-hour stage of development, that caused flies to have a diminished ability to survive at extremely hot temperatures (Figure 7a). One-hour flies at 30°C had a much lower upper thermal limit compared to the baseline. This shows that instead of acclimating flies to hot temperatures like 18°C flies were to cold temperatures, this thermal stress during early development disrupted the fly's ability to resist extremely hot temperatures. This disruption if heat stress was not applied until 24 hours was no longer present, with 30°C flies not having their ability to resist extreme temperatures be disturbed at this developmental stage (Figure 7b). However, 30°C was not

sufficiently hot enough to acclimate fruit flies to heat stress when heat was applied by 24 hours of development or earlier. Like with cold acclimation, it has been previously shown that moderate and chronic heat stress during development led to heat acclimation for survival at high temperatures during adulthood (Colinet et al. 2013). It is therefore surprising that no heat acclimation was found here and 24-hour 30°C flies had their upper thermal limit equal to the baseline, with no advantage over the 25°C group like 18°C flies did with CTmin. Heat stress during development has been shown to increase the protein abundance of several HSPs like Hsp70, Hsp60, and Hsp22, which contribute to heat acclimation (Colinet et al. 2013). The increased thermotolerance of the 24-hour flies is likely influenced by the increased HSP expression at this stage, but the lack of a heat acclimation provides evidence that for the Canton-S strain at 30°C, the degree of heat acclimation due to a buildup of HSPs from heat stress is canceled out by the deleterious effects of the heat stress on the fly.

Temperate versus tropical fly populations

With early thermal stress being very damaging to the development and performance of fruit flies, adaptation to reduce those effects should be acting to promote resilience against developmental thermal stress. Heat and cold both represent thermal stresses that trigger the production of HSPs and other chaperones to prevent accumulation of stress within the cell. For temperate populations, specifically, the canalization of temperate European populations to trigger a massive upregulation of genes associated with HSPs have allowed them to exhibit increased cold tolerance in comparison to tropical African populations (Heckel et al. 2016). With the robustness of the cold response in temperate populations resulting in an advantageous preparedness, temperate Vermont populations were expected to present evidence of thermal adaptation during early development against cold stress. Likewise, it was expected for tropical flies to fare better in resisting heat stress during development than temperate flies. This is because throughout their evolutionary history, in their present locations, tropical flies have experienced greater average temperatures annually than temperate flies, with this pattern of geographic distribution reflecting differences in their thermal adaptation (Ayrinhac et al. 2004). Tropical and temperate populations have been shown to adjust and utilize phenotypic plasticity to their experienced environments, with an adaptive response to heat and cold stress, respectively, resulting in superior upregulation of stress-resistant genes to exhibit increased

tolerance to heat and cold, respectively (Trotta et al. 2006). This higher temperature throughout the history of tropical populations, therefore, should better prepare the heat resistance mechanisms within these flies when facing heat stress. *Drosophila melanogaster* has been living in North America and the Pacific region for approximately 140 and 100 years, respectively, with both regions showing evidence of thermal adaptation in their environments (Keller, 2007; Agis and Schlötterer, 2001). Since all the populations tested originated from those regions, sufficient time should have occurred for the populations to have exhibited thermal adaptation. The results, however, proved inconclusive. No cold adaptation or advantage in temperate populations against cold stress was showcased for any of the phenotypes or performance results tested. For tropical populations, one of the populations did provide evidence for heat adaptation for several metrics, but with the other tropical population not providing evidence for heat adaptation during development, the uniformity of the tropical climate in causing thermal adaptation during development against heat stress is called into question.

The ability to develop to the pupal stage in the face of thermal stress requires adequate mechanisms in place to resist the stress, not allowing the vital genes and pathways behind pupal development to be disrupted. Climate type had no effect on the ability of flies experiencing cold stress to develop into pupae (Figure 8a). There was a sign of adaptation at 30°C, as tropical flies were better able to develop into pupae than temperate flies (Figure 8b). However, tropical and temperate flies were equally successful in developing into pupae at 32°C (Figure 8c). A study on another fruit fly species, *Ceratitis capitata*, comparing tropical, temperate, and sub-tropical populations of the species, found no difference in survival across the populations at the larval and pupal stages (Ricalde et al. 2012). With tropical flies having the advantage at 30°C, but neither climate type having an advantage at 18°C or 32°C, this provides evidence that survival to the pupal stage in *D. melanogaster* does not differ much between climates. As a cosmopolitan species, *D. melanogaster* must have the capacity and plasticity needed to survive the range of temperatures experienced around the globe, so survival to the pupal stage may not create sufficient evolutionary pressure to cause clear thermal adaptation between climates (Ricalde et al. 2012).

For eclosion success, as with many other phenotypes and performance values obtained, no clear evidence of heat or cold adaptation for all the tropical or all the temperate populations

were found, respectively. Comparing eclosion success between climate types, 18°C flies eclosed the same for temperate and tropical flies and tropical 32°C flies experienced a higher proportion of eclosion than temperate flies reared at 32°C (Figure 9). However, for this phenotype as well as many others that will be discussed, this advantage found in tropical flies is mainly driven by Chiapas flies and not Guam flies. Chiapas flies experienced the highest proportion of eclosion at 30°C and 32°C (Figure 10). However, Guam flies at 30°C experienced the worst proportion of eclosion of all the sites, and the same proportion of eclosion as VT8 at 32°C (Figure 10). Like with pupae survival, survival to adulthood in a related fruit fly species has been shown to not differ between tropical and temperate populations (Ricalde et al. 2012). *Drosophila melanogaster* could, therefore, have the necessary plasticity to adjust to changing environments and survive to adulthood.

It may be inappropriate to group Guam and Chiapas into the same category, as different factors could be at play in these two sites, which are found on opposite sides of the world. Even though the tropics are very consistent in temperature, perhaps mitigating factors like plant cover, humidity, and wind might make the temperature experienced by flies vary from place to place. The temperate populations used both originated from Vermont and likely experienced very similar levels of heat, shelter, humidity, and weather. Connected with the similarities of the Vermont populations in pupae and adult development as well as the following results shows that the temperate populations used are more likely to represent a uniform climate type than the tropical populations used.

No evidence of heat or cold adaptation were present for the ability of flies to climb. VT10 flies climbed better than Guam flies at all temperatures, Chiapas flies at 25°C and 30°C, and VT8 flies at 30°C (Figure 11). Chiapas flies climbed better than VT8 flies at 30°C, but VT8 flies climbed better than Guam flies at 25°C (Figure 11). There was no interaction effect of climate type and developmental temperature, providing evidence that the climate type of these flies was not very important in providing any adaptations in resisting thermal stress's ability to disrupt locomotion development (Figure 11). One caveat to these results, however, is that climbing proportion was assessed when flies were tested for their lower thermal limit. The column used for CTmin assays experienced several performance problems throughout the length of the

experiment. The low proportion of climbing for several groups, especially Guam flies, might have been impacted by the column conditions.

Thermal stress was largely insufficient to disrupt the mechanisms behind wing development for tropical and temperate populations. Climate type, development temperature, site, as well as all the interaction effects of climate type and site with developmental temperature, had no effect on the proportion of deformed wings (Figure 12). However, Chiapas flies reared at 32°C did experience a relatively high proportion of deformed wings, with 12% of Chiapas flies reared at 32°C developing deformed wings (Figure 12). They were the only population that had enough successfully eclosed adults at 32°C to perform CTmins and to be counted for deformed wing proportion, which is the likely explanation why this group was not determined different from the others. Unlike 1-hour flies of Canton-S, all the 1-hour populations reared at 30°C exhibited a negligible number of deformed wings. The genetic diversity found in the natural populations tested likely had an impact on their resistance to deformity-inducing thermal stress, which was not as prominent in the Canton-S population. Natural populations of *D. melanogaster* have been shown to be resistant to wing deformities due to thermal stress up to rearing temperatures of 30°C (Roberts and Feder, 1999). Once flies were reared at temperatures ranging from 30°C up to 40°C, proportion of wing deformities more than doubled (Roberts and Feder, 1999). Thirty degrees may not be enough to induce wing deformities in natural populations of *D. melanogaster*, with 32°C and higher possibly representing a turning point where wing deformities can start to accumulate.

It was found that temperate flies did not show evidence of adaptation for cold tolerance for the flies that were reared at 18°C. When comparing values for CTmin, all the populations responded similarly. CTmin values for 18°C, 25°C, and 30°C flies, respectively, were all equal across all the sites, except for 25°C VT10 flies having a greater CTmin than 25°C Guam and VT8 flies (Figure 13). This largely supports previous work on *D. melanogaster* populations (Ayrinhac et al. 2004). Temperate populations of *D. melanogaster* have been shown to experience greater levels of cold tolerance at low temperatures compared to tropical populations, but only 4% of the differences could be attributed to genetic latitudinal differences (Ayrinhac et al. 2004). Most of the variation could be attributed to adaptive phenotypic plasticity arising from the temperatures the different treatments were reared in (Ayrinhac et al. 2004). Tropical and

temperate flies both had lower CTmin values when reared at 18°C than 25°C or 30°C, showcasing the phenotypic plasticity within fly populations that allow flies reared at low temperatures to develop cold tolerance, regardless of climate type (Figures 13).

For upper thermal limit, a heat adaptation was found in Chiapas flies but not Guam flies. Chiapas flies had a higher CTmax at 30°C than all other sites, as well as a larger 25°C and 18°C CTmax than VT8 and VT10 flies, respectively (Figure 14). Guam flies, however, had the same 30°C CTmax as both temperate sites, and an equally bad 18°C value (Figure 14). Tropical flies, overall, had a higher CTmax than temperate flies, but just like with eclosion and climbing success, that trend was due to the exceptional performance and superiority of Chiapas flies, with Guam flies frequently performing as well or worse as the temperate sites. It has been shown that embryos of tropical populations exhibit a higher upper thermal limit than temperate embryos (Lockwood et al. 2018). Thermal adaptation may be more likely at this stage due to embryos being more immobile and thermally sensitive than adults. However, between tropical and temperate adults, there was no difference found for upper thermal limits (Lockwood et al. 2018). The thermal tolerance and resistance, the mobility, and the phenotypic plasticity of adult fruit flies make it questionable to the degree to which adults of *D. melanogaster* exhibit divergence in thermal tolerance between temperate and tropical regions for upper thermal limit.

Throughout the entire research process, there have been clues into how these experiments and set-ups could be improved upon and what possible future experiments could arise from this research. The vertical and horizontal testing apparatuses frequently presented with bubbles, leakages, and irregular heat ramping. The accuracy and reliability of the CTmax, CTmin, and climbing data could be improved upon with superior testing apparatuses with fewer performance errors. Utilizing larger stocks of populations could increase the genetic diversity found at each site. The Vermont sites were much more like each other than Chiapas and Guam were to each other. The geographic proximity of the Vermont sites likely influenced this trend, but representing a greater snapshot of these natural populations, especially the tropical populations, by increasing the amount of genetic diversity used for each site might decrease the prevalence of some discrepancies, as well as better illustrate trends between climate types.

One of the downsides of this work is that for most of the phenotypes observed, only two life stages and four populations were tested. Additional life stages should be investigated in the

future to further pinpoint the timing of development of wings as well as the timing of the acquisition of resistance to thermal stress, allowing for improved functioning as adults. Specifically, due to the majority of HSP accumulation occurring by twelve hours into development (Welte et al. 1993), an additional treatment of flies reared in abnormal temperatures starting at around thirteen hours into development could help show if a stress defense mechanism other than HSPs plays a prominent role in development. Controlling for the expression of important HSPs like Hsp70 by adding or removing copies of the Hsp70 gene and introducing the flies to moderate and chronic thermal stress early in development could provide evidence into the role of Hsp70 expression in thermotolerance for critical thermal temperatures and phenotypic results. Also, using more than two populations per climate type in the future will utilize more sites that experienced unique evolutionary histories. When disparities arise between the only two populations of a climate type you are testing, like with Chiapas and Guam, an overall trend and quality of that climate type is harder to ascertain or pinpoint. So, utilizing additional populations allows whatever trends that certain climate types possess to be more self-evident.

Except for pupae developmental at 30°C, later developmental stages of fruit flies proved to be better equipped to resist the effects of thermal stress. Twenty four-hour flies had better eclosion, climbing, wing, and CTmax success at 30°C than 1-hour flies. Also, greater pupae success at 18°C and acclimation of CTmin at 18°C further showcase the improvements made at both cold and heat advantages at the later developmental stage. Wings being unaffected by thermal stress at the 24-hour stage show how sufficient wing development as well as thermotolerance levels are developed by this point. It is evident how more susceptible fruit flies are as embryos compared to larvae.

Testing for eclosion success, climbing success, and CTmin, a general theme was found across these different phenotypic and performance observations for our natural populations. Chiapas routinely performed better for most if not all of the developmental temperatures, compared to all the other sites. Guam, on the other hand, routinely performed as good or worse than both temperate fly populations. Both Vermont populations performed very similar to each for the various phenotypic and critical temperature results. This was expected as they are populations that come from the same geographical area and the genetic diversity of the two populations should not be too different from one another. As described above, Guam and

Chiapas are much more different in location. The results indicate that Chiapas is well adapted for heat stress for a variety of phenotypes and performance metrics, routinely performing better than temperate sites. However, this trend being only prominent in one tropical population shows how additional tropical populations need to be tested to further clarify any trends across climate types. As discussed previously, the cosmopolitan species *D. melanogaster* requires a high degree of plasticity to be able to survive and have high fitness in a variety of environments around the world. Previous studies have showcased that pupae and adult survival as well as cold and heat tolerance in adulthood is highly dependent on plasticity and the abilities of flies to adjust to their environment, with genetic differences due to evolutionary adaptations playing a less prominent role. This puts into question the degree of evolutionary pressure that is in place that would lead to high adaptive potential for critical thermal temperatures and survivability at different life stages across climate types. Be it temperature, humidity, predation, shelter prevalence, or seasonal changes, differences between the two tropical sites are an indication that the classification "tropical" may indeed be too broad. The differences between sites such as Chiapas and Guam show that further detail and investigation is needed to adequately classify these populations and understand how evolution has affected each of them in preparation against thermal stress.

Early developmental stages of *D. melanogaster* are highly susceptible to thermal stress, especially heat stress, and will face new challenges as climate change proceeds around the globe. Flies experiencing thermal stress earlier in development pose a higher risk for pupae and adult mortality, wing and locomotion disruptions, and decreased survival at extreme temperatures. It has been shown that *D. melanogaster* exhibits a large potential for change in response to new environment. However, climate change in the future will cause shifts in geographic distribution of many populations around the world (Lockwood et al. 2018; Sherwood and Huber, 2010). The global effects of climate change over the next decades will put more stress on flies in securing locations for egg-laying away from the sun and thermal stress, which will become more dangerous as global warming continues to ramp. Chiapas flies have shown partial potential for thermal adaptation at early development stages. With it being questionable the degree to which populations of *D. melanogaster* have the evolutionary force needed to experience thermal adaptations in development, the existing plasticity within populations along with the increasing

pressures posed to populations by climate change may play a large role in the future fitness, performance, distribution, and survival of this model species around the globe.

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